DEVELOPMENT OF A BROAD-SPECTRUM OXIME FOR THE TREATMENT OF NERVE AGENT TOXICITY

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ABSTRACT

Inhibition of synaptic acetylcholinesterase (AChE) by organophosphate (OP) nerve agents is the main reason for their toxicity. Oximes are used as antidotes to reactivate nerve agent-inhibited AChE. To understand the mechanism of oxime-induced reactivation, we generated several mutant AChEs. Reactivation studies conducted with wild-type and mutant AChEs revealed that the peripheral anionic site of AChE plays a critical role in the reactivation of nerve agent-inhibited AChE by bis-pyridinium oximes, and not by mono-pyridinium oximes. Results showed that Y124 is an important determinant for the enhanced reactivation potency of HI-6 and HLo-7. Results also suggest that both the second pyridinium structure and the ether oxygen of HI-6 and HLo-7 are involved in interactions with the peripheral anionic site of AChE. These interactions are important considerations for the development of a next generation broad-spectrum oxime.

1. INTRODUCTION

OP nerve agents, such as sarin (GB), VX, soman (GD), cyclosarin (GF), and VR (see Fig. 1) are highly toxic chemical warfare agents. These agents pose significant threats to both military and civilian populations due to the increasing possibility of their use on the battlefield and in terrorist acts. The acute toxic effects of these OP nerve agents are due to the irreversible inhibition of synaptic AChE (EC 3.1. 1.7), whose function is to hydrolyze the neurotransmitter acetylcholine released at the pre-synapses and terminate signal transmission at the cholinergic synapses. This results in accumulation of acetylcholine in the synapses and over-stimulation of post-synaptic cholinergic receptors, which results in paralysis of neuromuscular function and a cholinergic crisis (Marrs, 1992). Depending on the extent of poisoning, these symptoms could develop very rapidly, resulting in death within several minutes.

To attenuate nerve agent toxicity, current medical countermeasure uses an oxime to reactivate OP-inhibited AChE. Since the 70's, the US Army has fielded the mono-pyridinium oxime 2-PAM as the antidote. However, the efficacy of 2-PAM is very limited against poisoning by some nerve agents such as GD and GF (Boskovich et al., 1984; de Jong and Kosen, 1985; Lundy et al., 1992; Luo and Liang, 1997; Worek et al., 1998). Subsequently more potent bis-pyridinium oximes TMB4 and obidoxime were studied. Unfortunately, they were not efficient reactivators of GD-inhibited AChE. In the 70's and 80's, HI-6 and HLo-7 (the H-series oximes) were synthesized, which were shown to be powerful reactivators of GD-inhibited AChE (Hagedorn et al., 1978, de Jong et al., 1989). Though highly effective in reactivating GD-inhibited AChE, HI-6 was ineffective in reactivating tabun-inhibited enzyme (Clement 1982). HLo-7 is some what effective in reactivating tabun-inhibited AChE and at the same time maintains the potency for reactivating GD-inhibited AChE. However, the extreme instability of HLo-7 in aqueous solutions prevents its use as an effective nerve agent antidote. Nerve agents consist of classic agents (GB, GD, VX, and tabun) or new structures obtained by alteration of classic ones. For example, GF was found in the stockpile during Iran-Iraq war, and VR (also called Russian VX) was developed by the former Soviet Union. Therefore, the next generation antidote should be a broad-spectrum oxime, which can reactivate AChE inhibited by all nerve agents.

The development of a broad-spectrum oxime, how ever, requires a better understanding of the mechanism of oxime-induced reactivation of AChE. Although it has been 30 years since the synthesis of the first H-series oxime HI-6, the mechanism for its potency in reactivating GD-inhibited AChE is still unknown. Nor is its inactivity against tabun-inhibited AChE known. An understanding of the mechanism of oxime reactivation is imperative for the rational design of the next generation broad-spectrum oxime.

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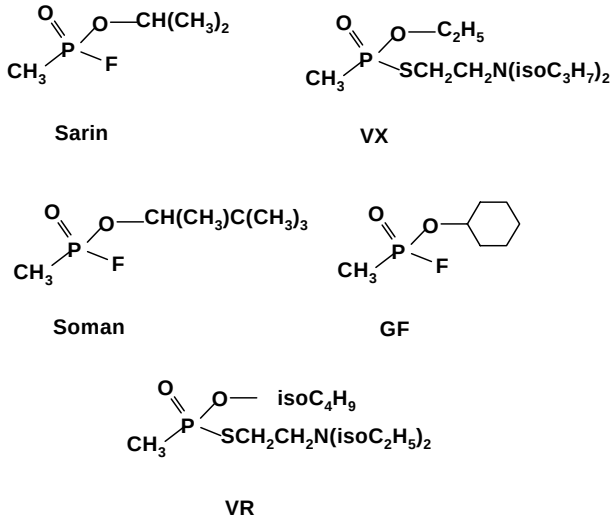

Fig. 1, Structures of organophosphate nerve agents.

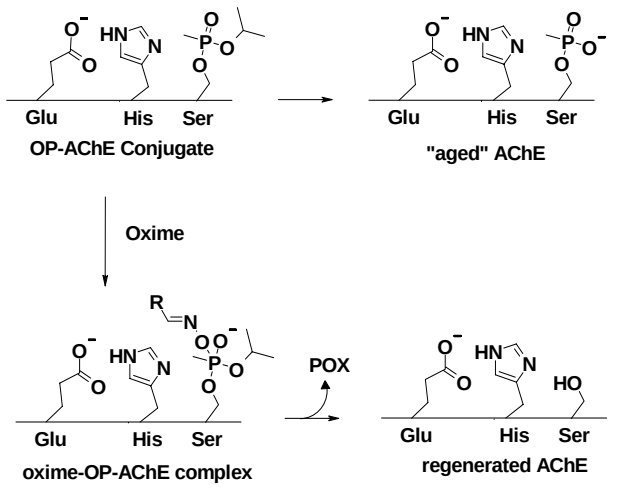

Fig. 2, Scheme for the reactivation of nerve agent-inhibited AChE by oxime. Without oxime, the inhibited AChE changes to aged AChE, which can no longer be reactivated by oxime.

As illustrated in Fig. 2, when AChE is inhibited by OP nerve agent, the γ-oxygen of the active-site serine forms a covalent bond with the phosphorus atom of the nerve agent residue. Oxime reactivates the inhibited enzyme by nucleophilic attack on the O—P bond of the nerve agent residue and generates free enzyme again. The nucleophilicity of the attacking group determines the effectiveness of the antidote. In addition, other structural elements facilitate the orientation of the attacking oxime group (Wilson et al., 1959). Mono-pyridinium oxime 2-PAM can successfully reactivate AChE inhibited by nerve agents containing small side-chain groups such as GB and VX, and OP pesticides such as paraoxon, because the quaternary pyridinium ring may help orient the oxime for neucleophilic attack. The low efficacy of 2-PAM against GD could be due to fast aging (dealkylation) of GD-inhibited AChE (de Jong and Wolring, 1985) or steric hindrance from the bulky pinacolyl side-chain, which precludes the proper orientation of the quaternary pyridinium ring of 2-PAM for nucleophilic attack. The finding that 2-PAM also demonstrates very low potency in reactivating GF-inhibited AChE suggests that steric hindrance may be the reason for 2-PAM's weak ability to reactivate both GD- and GF-inhibited AChE, since the cyclohexyl side-chain in GF is as bulky as the pinacolyl group in GD.

Understanding the mechanism of reactivation of GD- and GF-inhibited AChE by H-series oximes is an important step in the development of a broad-spectrum oxime. Previous reactivation studies with mutant mouse AChEs indicated that HI-6 may take two different orientations in the reactivation of AChE inhibited by methyl phosphonates and pesticide paraoxon (Luo et al., 2003). In a recent study we observed that HI-6 and HLo-7 demonstrated significantly higher potencies in reactivating GD-, GF-, and VR-inhibited AChE compared to GB- and VX-inhibited human AChE. Interestingly, VR is also an OP containing a relatively bulky *iso*-butyryl side-chain. It appears that the H-series oximes HI-6 and HLo-7 can overcome the steric hindrance imposed by these bulky side-chains and reactivate OP-inhibited AChE at a much faster rate compared to AChE inhibited by OPs containing smaller side-chains. In an effort to understand the mechanism underlying the high potency of H-series oximes, we conducted reactivation kinetic studies with single and multiple mutants of bovine (Bo) AChE generated by site-specific mutagenesis of desired amino acid residues. Due to the difficulty in measuring reactivation rate constants of rapidly aging GD-inhibited mutant AChEs, we studied reactivation of wild-type and different mutant Bo AChEs inhibited by GF and VR by five different oximes. Results shed light on the design and development of the next generation broad-spectrum oxime.

2. MATERIALS AND METHODS

2.1 Oximes Used in the Study

Oxime compounds 2-PAM, MMB-4, HI-6, HLo-7, and ICD-585 (see Fig. 3) were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research.

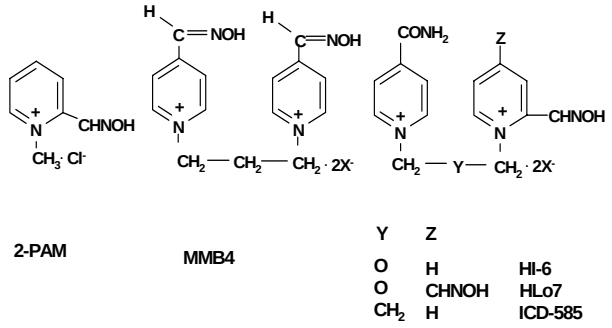

Fig. 3, Structures of mono-pyridinium and bis-pyridinium oximes used for the reactivation of nerve agent-inhibited recombinant wild-type and mutant bovine AChEs.

2.2 Production and Expression of Recombinant Bovine Wild-type and Mutant AChEs

Cloning of Bo AChE cDNA

Total RNA was isolated from calf brain using RNAzol B (Tel-Test, Inc (Friendswood, TX) according to the manufacturer's specifications. The coding sequence for the bovine (Bo) acetylcholinesterase (AChE) was obtained using RT-PCR amplification of the total RNA as specified by Applied Biosystems (Foster City, CA). Many PCR fragments were isolated and sub-cloned into TA vector pCR2.1 (Invitrogen, Carlsbad, CA) using the manufacturer's specifications. Two clones were chosen for further cloning efforts: one (which contained all of exon 2 minus the signal peptide sequence) and two (which contained approximately 70 base pairs of exon 2 sequence, but all of the sequences for exons 3, 4, and 6).

Construction of entire cDNA for mature T-subunit Bo AChE into vector pGEM7Z

Clone#1/pCR2.1 and clone#2/pCR2.1 were digested with restriction enzymes EcoRI and NcoI, and subcloned into the plasmid pGEM7Z as a single restriction fragment into the EcoRI site of the pGEM7Z vector. Colonies were screened for the proper orientation and insert size. A clone was obtained which contained the entire full length Bo AChE cDNA sequence in the correct orientation with the correct size in base pairs. Both strands of this clone were sequenced to determine its correctness. All restriction enzymes and other DNA modifying enzymes were purchased from Invitrogen. QIAquick Gel-Extraction Kit, Midi Plasmid DNA Isolation Kit, QIAquick PCR Purification Kit, and the QIAprep Spin Miniprep Kit were purchased from Qiagen (Valencia, CA). Plasmid pGEM7Z was purchased from Promega (Madison, WI).

Cloning Bo AChE cDNA into pCIneo mammalian expression vector

The signal peptide sequence of the Bo AChE was determined through PCR amplification of bovine genomic kidney DNA using degenerate oligos to the bovine signal peptide amino acid sequence and an oligo made from the coding sequence found at the end of exon 2 utilizing the high fidelity Pfx DNA polymerase from Invitrogen. Both strands of this PCR fragment were sequenced to determine its correctness. An EcoRI site was placed at the 5' end of the PCR fragment and an NcoI site was found near the end of exon 2. The PCR generated genomic DNA fragment (containing exon 2 and the bovine signal peptide sequence), and the Bo AChE cDNA (containing exons 3, 4, and 6) from the pGEM7Z clone were digested with the restriction enzymes (EcoRI and NcoI), fragmented by agarose gel electrophoresis, the desired DNA fragments were isolated, agarose gel DNA purified, and ligated together. Therefore, a continuous open reading frame coding for the Bo AChE T-subunit was subcloned into the EcoRI site of the pCIneo vector from Promega.

Sequencing

For sequence determination, the ABI PRISM Big Dye version 3 Ready Reaction Terminator Cycle Sequencing Kit from Applied Biosystems (Foster City, CA) was purchased. It was utilized according to the manufacturer's instructions and run on the ABI 3100 Genetic Analyzer. Both strands of the Bo AChE cDNA construct were sequenced using the appropriate primers derived from the nucleotide sequence. Each sequence was compared (using BLAST) with the known bovine AChE sequences in GenBank database under accession numbers AF061813, AF061814, AF061815, and AF061816 representing exons 2, 3, 4, and 6, respectively.

Transfection, Transient Expression and Establishment of Stable cell lines in eukaryotic CHO-K1 cells

Lipofectamine 2000 reagent was purchased from Invitrogen and used as the transfection reagent. The mammalian cells chosen for the transfection studies were CHO-K1 epithelial cells (CCL-61) obtained from the ATCC (Manassas, VA). CHO-K1 cells were transfected using the pCIneo vector containing the Bo AChE cDNA at a concentration of 1 to 5μg of DNA to 1x 10^5 cells per well in a 24-well culture plate. The day before performing transfection of the CHO-K1 cells, the cells were trypsinized, counted, and plated to a density to yield 90-95% confluence. The transfection experiments were conducted according to the specifications of the manufacture Invitrogen. Transient expression was assayed 24 and 48 hours post transfection using the Ellman method. The CHO-K1 cells that contain the pCIneo/Bo AChE construct express the neomycin phosphotransferase gene which confers resistance to the

antibiotic G-418. Establishment of a stable cell line was accomplished by selection using the antibiotic geneticin (G-418). The transfected CHO-K1 cells that contained the plasmid pCIneo/Bo AChE cDNA secrete the recombinant Bo AChE enzyme into the culture medium. The culture medium, Ultraculture, was purchased from Biowhittaker (distributed by Fisher Scientific, Pittsburgh, PA).

Mutagenesis of recombinant bovine AChE

Site-directed mutagenesis was conducted using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). PCR was performed using pairs of forward and reverse mutagenic oligonucleotide primers. The desired mutation occurred at the same position but on opposite strands of the plasmid (pCIneo/ Bo AChE). The oligonucleotides were synthesized at USUHS (Bethesda, MD). The mutation in the pCIneo/ Bo AChE cDNA was created according to the QuikChange II XL Site-Directed Mutagenesis Kit protocols. After site-directed mutagenesis was completed, colonies were picked, grown as minipreps, and their DNAs were isolated. All potential clones were sequenced. Once the mutation was identified, the entire 1.8kb bovine insert was sequenced to confirm no spurious changes had occurred. In the case of the quadruple mutant, it was necessary to make each change separately before proceeding to the next mutation, therefore, a double mutant Y72N/D74G was constructed, then Y124Q was added, and finally W286A was added to the construct to produce the quadruple mutant Y72N/D74G/Y124Q/W286A. Each new mutant DNA clone was used as previously described to perform transfection of CHO-K1 cells, transient expression, and establishment of a stable cell line. Each mutant Bo AChE was established as a stable cell line that secreted the recombinant enzyme into the Ultraculture media. The mutant enzymes were collected and purified.

Recombinant wild-type and mutant Bo AChEs expressed in the cell media of CHO cells were purified by procainamide-sepharose 4B affinity chromatography (De La Hoz et al., 1986) followed by DEAE anion exchange chromatography in 50 mM sodium phosphate buffer, pH 8.0. The (Y72N/D74G/Y124Q/W286A) quadruple mutant AChE did not bind to procainamide-sepharose 4B gel and the tetrameric form of this enzyme was partially purified by gel filtration chromatography on a 1.5 M Bio Gel A column (Bio-Rad, CA).

2.3 Assay for AChE Activity

AChE activity was determined by the Ellman method (Ellman et al., 1961). The assay mixture contained 1 mM acetylthiocholine as the substrate and 0.5 mM 5,5-dithiobis-(2-nitro-benzoic acid) in 50 mM sodium phosphate buffer, pH 8.0. The formation of product was measured at 412 nm at 25° C.

2.4 Determination of the Reactivation Rate Constants of Nerve agent-Inhibited AChEs by oximes

The oxime reactivation rate constants were determined by spectrophotometric monitoring of the reactivation of the OP-inhibited AChE as described (Wong et al., 2000). To minimize the aging of OP-inhibited AChE, the enzyme conjugates were prepared in 100 mM HEPES, pH 9.5. Excess OP was removed from the conjugate on a Bio-Spin column (Bio-Rad, CA) or neutralized by adding more enzyme to the solution. The enzyme conjugates prepared at USAMRICD, were transported to WRAIR and stored at -20°C in 50% glycerol, until further use. Oxime reactivation was examined at 25° C in 50 mM sodium phosphate buffer, pH 8.0, containing 0.05% bovine serum albumin (BSA) and an oxime at a final concentration ranging from 0.125 μM to 5 mM., Aliquots of 5-25 μL of reactivation mixture were withdrawn at specified time intervals and diluted into the assay mixture to monitor change in enzyme activity. The pseudo first-order reactivation rate constant, k_{obs}, was determined by fitting the experimental data to the equation for one phase exponential association:

$$\%\,(E_{react})_t = A(1 - e^{-kobs \cdot t}) \quad (1)$$

where t is the time at which the sample was withdrawn; $\%(E_{react})_t$ is percent reactivation measured at time t, and A is percent of maximum reactivation measured after 24 h incubation.

A secondary plot of k_{obs} versus [oxime] was used to obtain the second-order reactivation rate constant:

$$k_{obs} = k_2\,(1 + K_{ox}/[\text{oxime}] \quad (2)$$

$$k_r = k_2 / K_{ox} \quad (3)$$

where k_2 is the intrinsic reaction constant; [oxime], is the concentration of oxime used; K_{ox}, is the apparent equilibrium constant; k_r, is the second-order reactivation rate constant. In some cases the secondary plot of k_{obs} versus [oxime] was a straight line instead of a saturation curve. In these cases, only k_r was estimated from the slope of the line.

3. RESULTS

In this study, five different oximes were examined for their ability to reactivate GF- and VR-inhibited wild-type and mutant Bo AChEs. Examples for the determination of second-order reactivation rate constants

are shown in Figs. 4 and 5. In Fig.4, the plot of pseudo first-order reactivation rate constant (k_{obs}) vs [MMB4] yielded a saturation curve, which enabled the determination of K_{ox} and k_2. The second-order reactivation rate constant k_r was calculated by dividing k_2 by K_{ox}. In Fig. 5, the plot of k_{obs} vs [HLo-7] was linear. In this case, K_{ox} and k_2 could not be determined and k_r was estimated from the slope of the line. The second-order reactivation rate constants of GF- and VR-inhibited recombinant wild-type and various mutant Bo AChEs by five oximes are summarized Tables 1 and 2. Results show that reactivation rate constants for wild-type Bo AChE inhibited by these two nerve agents are quite similar, with the following rank order for the potency of five oximes: (HLo-7>HI-6>ICD-585>MMB4>2-PAM). The replacement of aromatic or negatively charged amino acid residues in wild-type enzyme by aliphatic or neutral ones resulted in significant changes in reactivation rate constants. Both GF- and VR-inhibited mutant AChEs showed similar effects on reactivation, except for D74G AChE. The reactivation rate constants for GF-inhibited D74G AChE by MMB4 and ICD-585 were 0.6 and 4.1-fold higher, but reactivation of VR-inhibited AChE were 2.2 and 0.6-fold lower, compared to wild-type AChE.

Of the five single mutant AChEs examined, the active site mutant Y337A showed higher oxime reactivation rate constants compared to wild-type enzyme in most cases. Increases in reactivation rates were also observed for the peripheral anionic site mutant D74G. The other three peripheral anionic site mutants (Y72N, Y124Q, and W286A) showed reductions in oxime reactivation rates in most cases. W286A had the least effect with 6- and 1-fold reduction in the rate constant for the reactivation of GF- and VR-inhibited enzyme, by 2-PAM, respectively, and less than 2-fold reduction in the reactivation rate for all bis-pyridinium oximes. Mutation of Y72 to N resulted in reduction in reactivation by bis-pyridinium oximes from 1 to 9-fold, but had no effect on reactivation by 2-PAM. The most significant effect of a single mutation was observed on the reactivation of GF- and VR-inhibited Y124Q AChE by HI-6 and HLo-7. The reactivation rate constants of GF- and VR-inhibited Y124Q AChE were reduced from 16 to 31-fold compared with that of wild-type Bo AChE. However, the effect of this mutation on reactivation by the other two bis-pyridinium oximes were much less significant (3 to 4-fold reduction), and no changes were observed for mono-pyridinium oxime 2-PAM.

The most dramatic changes observed were with a quadruple mutant (Y72N/D74G/Y124Q/W286A) AChE, in which the four anionic and aromatic amino acid residues in the peripheral anionic site of the enzyme, were replaced with aliphatic residues. The reactivation of this GF- and VR-inhibited mutant enzyme by all the four bis-pyridinium oximes was at least 180 to 1500-fold slower compared to wild-type AChE. However, only a 3 fold reduction was observed for the reactivation of GF- and VR-inhibited quadruple mutant AChE by mono-pyridinium oxime 2-PAM.

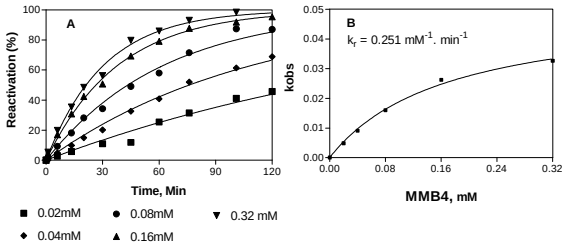

Fig. 4, Reactivation of cyclosarin-inhibited wild-type bovine AChE by MMB4. **A**, plot of percent reactivation vs. time to obtain k_{obs} for each oxime concentration; **B**, plot of k_{obs} vs. oxime concentration to obtain k_r.

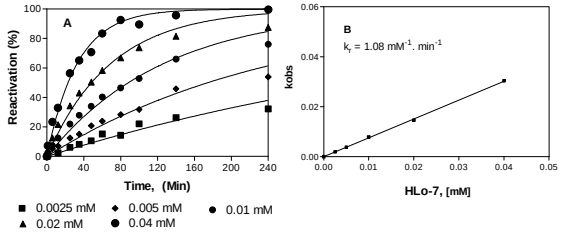

Fig. 5, Reactivation of VR-inhibited Y72N bovine AChE by HLo-7. **A**, plot of percent reactivation vs. time to obtain k_{obs} for each oxime concentration; **B**, slope of the linear line in plot of k_{obs} vs. oxime concentration to estimate k_r.

4. DISCUSSION

The reactivation of GF- and VR-inhibited wild-type Bo AChE by five oximes displayed the same rank order for potency of oximes. This observation indicates that the two OP-AChE conjugates share some similarity in the reactivation mechanism. All four bis-pyridinium oximes were significantly better than the mono-pyridinium oxime 2-PAM, indicating that the additional pyridinium structure facilitated reactivation by bis-pyridinium oximes. Reactivation rates for H-series oximes HI-6 and HLo-7 were significantly higher compared to those for other bis-pyridinium oximes, which is in agreement with previously published reports (Worek et al., 1998). The 180- to 1500-fold reductions in the reactivation rates of GF- and VR-inhibited quadruple mutant by all bis-pyridinium oximes suggest that the peripheral anionic site of the enzyme plays an important role in reactivation by bis-pyridinium oximes and not mono-pyridinium

Table 1. Second-order Rate Constants ($M^{-1}.min^{-1}$) for the Reactivation of Gf-inhibited Recombinant Bovine Wild-type and Mutant AChEs by Oximes

Oxime	Wild-type	Y72N	D74G	Y124Q	W286A	Y337A	Y72N/D74G/Y124Q/W286A
2-PAM	56	77	15	43	8	68	13*
MMB4	251	24	410	49	138	203	1.4*
HI-6	1,344	482	9,990	71	471	17,970	1.9*
HLO-7	3,413	909	53,570	195	1,892	52,380	2.3*
ICD-585	467	347	2,388	131	478	8,219	2.0*

k_rs were obtained from at least 2 repeated kinetic determinations at 25 °C in 50 mM sodium phosphate buffer, pH 8.0, containing 0.05% BSA. * Only one determination due to the limited availability of this mutant.

Table 2. Second-order Rate Constants ($M^{-1}.min^{-1}$) for the Reactivation of VR-inhibited Recombinant Bovine Wild-type and Mutant AChEs by Oximes

Oxime	Wild-type	Y72N	D74G	Y124Q	W286A	Y337A	Y72N/D74G/Y124Q/W286A
2-PAM	78	79	2.3	79	42	61	18*
MMB4	870	105	270	224	796	780	1.5*
HI-6	2,192	419	9,038	68	1,052	8,072	2.7*
HLO-7	3,891	1,080	10,835	234	1,648	7,309	5.4*
ICD-585	895	297	543	221	1,017	4,380	1.1*

k_rs were obtained from at least 2 repeated kinetic determinations at 25 °C in 50 mM sodium phosphate buffer, pH 8.0, containing 0.05% BSA. * Only one determination due to the limited availability of this mutant.

oxime. In fact, the reactivation rate constants for GF- and VR-inhibited quadruple mutant by all four bis-pyridinium oximes were several fold lower than that for 2-PAM. A previous study using recombinant mouse AChE inhibited by MEPQ, an analog of nerve agent VX, demonstrated a similar though less dramatic reduction in HI-6-induced reactivation of a peripheral anionic site triple mutant AChE (Ashani et al., 1995). These results indicate that the second-pyridinium structure of bis-pyridinium oximes facilitates the nucleophilic attack of the oxime group by interacting with the peripheral anionic site of the enzyme. With the loss of these interactions as in the quadruple mutant AChE, the bis-pyridinium oximes are unable to attain proper orientation for nucleophilic attack. This may explain the slower reactivation of quadruple mutant AChE by all bis-pyridinium oximes compared with the mono-pyridinium oxime 2-PAM.

Results of single amino acid mutations at the peripheral anionic site revealed that single changes affected reactivation moderately. Of all the four bis-pyridinium oximes tested, the most significant reductions were observed with only Y124 Q AChE when HI-6 and HLo-7 were used as reactivators, suggesting that Y124 plays an important role in reactivation by these two H-series oximes. Due to steric hindrance, reactivation of AChE inhibited by nerve agents containing bulky side-chains, such as GD, GF, and VR, is more difficult than that inhibited by OPs containing less bulky side chains, such as GB and VX. However, H-series oximes are able to overcome this steric hindrance and are even better reactivators of AChE inhibited by OPs containing bulky side-chains. Once Y124 was changed to Q, the advantage of HI-6 and HLo-7 over the other two bis-pyridinium oximes was lost, since the reactivation rate constants for both GF- and VR-inhibited Y124Q AChE by all four bis-pyridinium oximes are almost similar. In fact the reactivation of VR-inhibited Y124Q AChE by HI-6 was even slower than by MMB4 and ICD-585. The difference between HI-6 and ICD-585 is that the ether oxygen in HI-6 is replaced by a methylene group in ICD-585, which reduces the reactivation potency of ICD-585 compared to HI-6. Therefore, the enhanced reactivation potency of H-series oximes can be attributed to both the second-pyridinium ring and the ether oxygen. The interaction of these structural elements with Y124 at the peripheral anionic site plays an important role in the reactivation of AChE inhibited by nerve agents containing bulky side-chains.

The loss of negative charge as in D74G AChE, is expected to weaken the interaction with the positively charged oxime, only reduced the reactivation of GF- and VR-inhibited D74G AChE by 2-PAM, 3 and 33-fold, respectively. It increased reactivation by bis-pyridinium oximes in most the cases, indicating that this amino acid at the peripheral anionic site is not important for reactivation by bis-pyridinium oximes. The active-site mutant Y337A AChE also showed an increase in oxime

reactivation in most cases. This result is in agreement with a previous study with mouse AChE which demonstrated that mutation of the aromatic residue in the active site to a smaller aliphatic residue enlarges the available space for bis-pyridinium oximes and increases the efficiency of oxime reactivation (Zrinka et al., 2004).

In summary, the results of this study on oxime reactivation of wild-type and mutant Bo AChEs revealed that the peripheral anionic site of AChE plays a critical role in the reactivation of nerve agent-inhibited AChE by bis-pyridinium oximes, but not mono-pyridinium oximes. Y124 is an important determinant for the potent reactivation ability of HI-6 and HLo-7. Both the second pyridinium structure and the ether oxygen of HI-6 and HLo-7 interact with amino acid residues at the peripheral anionic site of AChE to facilitate reactivation, and are important structural elements that should be considered in the development of a next generation broad-spectrum oxime.

REFERENCES

Ashani, Y., Radic, Z., Tsigelny, I., Vellom, D.C., et al., 1995: Amino Acid Residues Controlling Reactivation of Organophosphonyl Conjugates of Acetylcholineesterase by Mono- and Bis-quaternary Oximes, *J. Biol. Chem.*, **270**, 6370-6380.

Boskovic, B., Kovacevic, V., Jovanovic, D., 1984: PAM-2 Cl, HI-6, and HGG-12 in Soman and Tabun Poisoning, *Fundam. Appl. Toxicol.*, **4**, S106-115.

Clement, J.G. 1982: HI-6: Reactivation of Central and Peripheral Acetylcholinesterase Following Inhibition by Soman, Sarin and Tabun in vivo in the Rat, *Biochem. Pharmacol.*, **31**, 1283-1287

De La Hoz, D., Doctor, B.P., Scott, J., Ralston, J.S., et al., 1986: A Simplified Procedure for the Purification of Large Quantities of Fetal Bovine Serum Acetyl-cholinesterase, *Life Sci.*, **39**, 195-199.

De Jong**,** L.P., and Kossen, S.P., 1985: Stereospecific Reactivation of Human Brain and Erythrocyte Acetylcholinesterase Inhibited by 1,2,2-trimethyl-propyl methylphosphonofluoridate (soman), *Biochim. Biophys*. Acta. **830**, 345-348.

D Jong L.P., and Wolring, G.Z., 1985: Aging and Stereospecific Reactivation of Mouse Erythrocyte and Brain Acetylcholinesterase Inhibited by 1,2,2-trimethyl-propyl methylphosphonofluoridate (soman), *Biochem. Pharmacol*., **34**, 142-145.

De Jong, L.P.A., Verhagen, M.A.A., Langenberg, J.P., Hagedorn I., et al., 1989: The Bispyridinium-dioxime HLo-7: A Potent Reactivator for Acetylcholinesterase Inhibited by the Stereoisomers of Tabun and Soman, *Biochem. Pharmacol.*, **38**, 633-640.

Ellman, G.L., Courtney, K.D., Andres, V., and Featherstone, R.M., 1961: A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity, *Biochem. Pharmacol.*, **7**, 88-95.

Hagedorn, I., Schoene, K., Schenkel, H., 1978: Reacttivierung Phosphorylierter Acetylcholinesterase: Insomere Bisquartare Salze von Pyridine-aldoxime Salts, *Arzneim Forsch*, **28**, 2055-2057.

Kovarik, Z., Radic, Z., Berman, H. A., Simeon-Rudolf, V., et al., 2004: Mutant Cholinesterases Possessing Enhanced Capacity for Reactivation of Their Phosphonylated Conjugates, *Biochemistry*, **43**, 3222-3229.

Lundy, P.M., Hansen, A.S., Hand, B.T., Boulet, C.A., 1992: Comparison of Several Oximes against Poisoning by Soamn, Tabun and GF, *Toxicol*., **72**, 99-105.

Luo, C. and Liang, J., 1997: Evaluation of Combined Toxic Effects of GB/GF and Efficacy of Jielin Injection against Combined Poisoning in Mice, *Toxicol. Lett.*, **92**, 195-200.

Luo, C., Leader, H., Radic, Z., Maxwell, D.M., 2003: Two Possible Orientations of HI-6 Molecule in the Reactivation of Organophosphate-inhibited Acetylcholinesterase, Biochem. Pharmacol. 66, 387-392.

Marrs T.C., 1992: Clinical and Experimental Toxicology of Organophosphates and Carbamates, Oxford, Butterworth & Heinemann; p., 555-577.

Wilson I.B., 1959: Molecular Complementary and Antidotes for Alkylphosphate Poisoning, *Fed. Proc.*, **18**, 752-758

Worek**,** F., Eyer, P., Szinicz, L., 1998: Inhibition, reactivation and aging kinetics of cyclohexylmethylphosphonofluoridate-inhibited human cholinesterases. *Arch. Toxicol.* **72**, 580-587.

Wong, L., Radic, Z., Bruggemann, R.J.M., Hosea, N., et al., 2000: Mechanism of Oxime Reactivation of Acetylcho-linesterase Analyzed by Chirality and Mutagenesis, *Biochemistry*, **39**, 5750-5757.